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(54) Title: HYALURONIC ACID MIMICS AND METHODS RELATED THERETO

(57) Abstract

HA mimics and methods related thereto are disclosed. In particular, mimics with structures determined by virtue of novel methods, and the novel methods are disclosed. The HA mimics are useful for a variety of HA-related uses.

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HYALURONIC ACID MIMICS AND METHODS RELATED THERETO

This application claims priority to U.S. Provisional Patent Application Serial Number 60/091,758, filed July 6, 1998.

FIELD OF THE INVENTION

This invention pertains to the fields of biochemistry, specifically biochemistry related to compounds which interact with hyaluronic acid receptors.

BACKGROUND OF THE INVENTION

Hyaluronic acid (HA) is a large glycoaminoglycan that contains repeating disaccharide units of N-acetyl glucosamine and glucuronic acid. It occurs in the extracellular matrix and on the cell surface. It has been shown, among other things, to promote cell mobility, adhesion, and proliferation. HA has an role in many physiological processes, for example, morphogenesis, wound repair, inflammation, and metastasis. Many of the effects of HA are mediated through cell surface receptors, several of which have been molecularly characterized, namely CD44, RHAMM (Receptor for Hyaluronan Mediated Mobility), and ICAM- I (Intracellular Adhesion Molecule- 1), BEHAB, Link Protein and TSG-6. Binding of the HA ligand to its receptors triggers signal transduction events.

Although considerable information on the structure of the HA-related surface receptors is available, the three-dimensional structure/biochemistry of HA which influences the receptor/HA interaction is not known. The popular hypothesis for receptor-HA interaction is that the HA binding motif is present in sequences of these receptors and is responsible for the binding of these sequences to HA.

Other journal articles which hypothesize motifs that would bind to HA binding domains are:

Day, A. J. (1999) The structure and regulation of hyaluronan-binding proteins. Biochem Soc Trans 27, 115-21.

Knudson, C. B., and Knudson, W. (1993) Hyaluronan-binding proteins in development, tissue homeostasis, and disease. Faseb J 7, 1233-41

Sherman, L., Sleeman, J., Herrlich, P., and Ponta, H. (1994) Hyaluronate receptors: key players in growth, differentiation, migration and tumor progression. Curr Opin Cell Biol 6, 726-33.

Toole, B. P. (1990) Hyaluronan and its binding proteins, the hyaladherins. Curr Opin Cell Biol 2, 839-44.

Ward, A. C., Dowthwaite, G. P., and Pitsillides, A. A. (1999) Hyaluronan in joint cavitation. Biochem Soc Trans 27, 128-35.

Bajorath, J., Greenfield, B., Munro, S. B., Day, A. J., and Aruffo, A. (1998). Identification of CD44 residues important for hyaluronan binding and delineation of the binding site. J Biol Chem 273, 338-43.

Kohda, D., Morton, C. J., Parkar, A. A., Hatanaka, H., Inagaki, F. M., Campbell, I. D., and Day, A. J. (1996). Solution structure of the link module: a hyaluronan-binding domain involved in extracellular matrix stability and cell migration. Cell 86, 767-75.

Maier, R., Wisniewski, H. G., Vilcek, J., and Lotz, M. (1996). TSG-6 expression in human articular chondrocytes. Possible implications in joint inflammation and cartilage degradation. Arthritis Rheum 39, 552-9.

Parkar, A. A., Kahmann, J. D., Howat, S. L., Bayliss, M. T., and Day, A. J. (1998). TSG-6 interacts with hyaluronan and aggrecan in a pH-dependent manner via a common functional element: implications for its regulation in inflamed cartilage. FEBS Lett 428, 171-6.

The following patents discuss modified HAs, none of which are remotely similar to the present HA mimics:

5,874,417 & 5,616,568 Functionalized derivatives of hyaluronic acid

5,652,347 Method for making functionalized derivatives of hyaluronic acid

5,631,241 Pharmaceutical compositions containing hyaluronic acid fractions

5,356,883 & 5,502,081 & 5,356,883 & 5,017,229 & 4,937,270 Water-insoluble derivatives of hyaluronic acid and their methods of preparation and use

5,520,916 Non-woven fabric material comprising hyaluronic acid derivatives and use

5,503,848 Spongy material consisting essentially of hyaluronic acid or its derivatives, and its use in microsurgery

5,202,431 Partial esters of hyaluronic acid

4,636,524 & 4,605,691 & 4,582,865 Cross-linked gels of hyaluronic acid and products containing such gels

The following patents discuss the use of HA for treating various diseases, which uses are also applicable uses of the present HA mimics:

5,914,314 Use of a form of hyaluronic acid and a medicinal agent for reducing rejection of organs transplantation in mammals

5,888,986 & 5,880,108 5,591,724 Method for treating the urinary bladder and associated structures using hyaluronic acid

5,847,002 Compositions, for inhibition, control and regression of angiogenesis, containing hyaluronic acid and NSAID

5,834,444 Hyaluronic acid and salts thereof inhibit arterial restenosis

5,830,882 Compositions containing a form of hyaluronic acid and a medicinal agent for treating acne in mammals and methods for administration of such composition

5,827,834 Method of using hyaluronic acid or its pharmaceutically acceptable salts for the treatment of disease

5,728,391 Hyaluronic acid and its salt for treating skin diseases

5,679,655 Method of treating lesions resulting from genital herpes with hyaluronic acid-urea pharmaceutical compositions

5,674,857 Use of hyaluronic acid to repair ischemia reperfusion damage

5,646,129 Method of using low molecular weight hyaluronic acid for stimulating bone formation

5,639,738 Treatment of basal cell carcinoma and actinic keratosis employing hyaluronic acid and NSAIDs

5,633,003 Use of intratracheally administered hyaluronic acid to ameliorate emphysema

5,631,242 Hyaluronic acid-urea pharmaceutical compositions utilized for treatment of diseases of cutis

5,624,915 & 5,583,120 & 5,583,119 & 5,550,112 & 5,529,987 Hyaluronic acid-urea pharmaceutical compositions and uses

5,614,506 Use of hyaluronic acid and forms to prevent arterial restenosis

5,604,200 Wound therapeutic mixture containing medical grade hyaluronic acid and tissue culture grade plasma-fibronectin in a delivery system that creates a moist environment which simulates in utero healing

5,583,118 Method of treating an anorectal disease using hyaluronic acid-urea pharmaceutical compositions

4,801,619 Hyaluronic acid preparation to be used for treating inflammations of skeletal joints

The following patents have disclosed compositions of matter comprising HA as an ingredient, and which would be useful in making the present compositions claimed, except that the present HA mimics would be substituted for HA:

5,847,002 Compositions, for inhibition, control and regression of angiogenesis, containing hyaluronic acid and NSAID

5,679,655 Method of treating lesions resulting from genital herpes with hyaluronic acid-urea pharmaceutical compositions

The following patents have disclosed other methods of using HA, which other uses would be applicable uses of the present HA mimics:

5,772,982 Method of using hyaluronic acid for the detection, location and diagnosis of tumors

4,804,537 Sperm selection process using a salt of hyaluronic acid

Citation of the above documents is not intended as an admission that any of the foregoing is pertinent prior art. All statements as to the date or representation as to the contents of these documents is based on subjective characterization of information available to the applicant, and does not constitute any admission as to the accuracy of the dates or contents of these documents.

SUMMARY OF THE INVENTION

The present invention provides HA mimics comprising a polypeptide having alternating acidic / non-acidic residues, wherein said mimic binds to the hyaluronic acid binding domain of the receptor for hyaluronan mediated mobility. Those HA mimics wherein said polypeptide is four to 15 residues in length are preferred. More preferred are those HA mimics as described, wherein said residues include at least one dextrorotatory residue. Most preferred are HA mimics comprising an amino acid sequence selected from the group consisting of: SEQ ID NO 1; SEQ ID NO 2; SEQ ID NO 3; SEQ ID NO 4; SEQ ID NO 5; SEQ ID NO 6; SEQ ID NO 7; SEQ ID NO 8; SEQ ID NO 9; SEQ ID NO 10; SEQ ID NO 11; SEQ ID NO 12; SEQ ID NO 13; SEQ ID NO 14; SEQ ID NO 15; SEQ ID NO 16; SEQ ID NO 17; SEQ ID NO 18; SEQ ID NO 19; and SEQ ID NO 20, or a homologue thereof, wherein said homologue binds to the hyaluronic acid binding domain of the receptor for hyaluronan mediated mobility.

In a preferred embodiment of the present invention, the HA mimics comprising the sequence:

$$X\text{-}A/a\text{-}X\text{-}A/a\text{-}X\text{-}a\text{-}X\text{-}a_1$$

wherein (X) can be any amino acid, (A) is L-glutamate or L-aspartate and (a) and (a_1) are D-glutamate or D-aspartate.

Those wherein X is hydrophobic are more preferred and those wherein X is hydrophobic and a_1 is D-glutamate are most preferred.

The present invention also provides nucleic acid compounds comprising a nucleic acid which encodes an amino acid sequence selected from the group consisting of: SEQ ID NO 1; SEQ ID NO 2; SEQ ID NO 3; SEQ ID NO 4; SEQ ID NO 5; SEQ ID NO 6; SEQ ID NO 7; SEQ ID NO 8; SEQ ID NO 9; SEQ ID NO 10; SEQ ID NO 11; SEQ ID NO 12; SEQ ID NO 13; SEQ ID NO 14; SEQ ID NO 15; SEQ ID NO 16; SEQ ID NO 17; SEQ ID NO 18; SEQ ID NO 19; and SEQ ID NO 20; and homologues thereof, wherein said homologue binds to the hyaluronic acid binding domain of the receptor for hyaluronan mediated mobility.

Also provided are HA mimics comprising a polypeptide comprising double hydrophobic residues, wherein said mimic binds to the hyaluronic acid binding domain of the TSG-6. In particular, provided are HA mimics of which are selected from the group consisting of: GYYFNVAM; WAYNFLVM; TQSLNNHM; WWPFINAY; WWKADMVG; WWPFINAY; MALQLPYY; IIYEEFFV; ISINNRWY; VTPPVYFT; QIRNGWFW; SWWFGPLA; GDWEQILT; PAGFGWNL; NMRFNIEN; and QMTFFDGV, or a homologue thereof, wherein said homologue binds to hyaluronic acid binding domain of the TSG-6. Nucleic acids which encode the above compounds are also part of the present invention.

The present invention also provides methods to affect a hyaluronidase-mediated biological response in a patient in need of such affecting , comprising administering an HA mimic of the present invention.

The present invention also provides methods to inhibit hyaluronidase activity in a patient in need of such inhibition, comprising administering an HA mimic of the present invention.

The present invention also provides methods to bind receptors that bind hyaluronic acid, comprising introducing an HA mimic of the present invention.

The present invention also provides methods to mimic hyaluronic acid in a biological system, comprising introducing an HA mimic of the present invention.

The present invention also provides methods to affect cell signalling associated with hyaluronic acid/hyaluronic acid receptor interactions, comprising introducing an HA mimic of the present invention.

The present invention also provides methods to treat hyaluronic acid-associated disease in a patient in need of such treatment, comprising administering an HA mimic of the present invention. In particular, those methods wherein said disease is selected from the group consisting of: inflammation; tumor angiogenesis; skin disease; bone disease; wound healing; osteoarthritis; rheumatoid arthritis; infectious disease; immune disease; and cardiovascular disease are preferred.

The present invention also provides methods to inhibit metastasis in a patient in need of such inhibition, comprising administering an HA mimic of the present invention.

The present invention also provides methods to inhibit fertilization in a patient in need of such inhibition, comprising administering an HA mimic of the present invention.

The present invention also provides methods to introduce an agent into a cell which binds hyaluronic acid, comprising administering the agent in conjunction with an HA mimic of the present invention.

The present invention also provides methods to isolate peptides that mimic a ligand's binding to a receptor comprising the steps of:

(a) preparing a random library of peptides and binding said library to a bead;

(b) placing the library in contact with the receptor under conditions for binding;

(c) washing off unbound peptides;

(d) contacting the washed bead-bound library with anti-receptor antibody;

(e) detecting and selecting beads having antibody bound thereto;

(f) eluting the antibody;

(g) repeating steps (a) through (f) and then repeating steps (a) through (c), and then incubating the beads in the presence of receptor prior to adding anti-receptor antibody;

(h) detecting and selecting beads that did not bind antibody;

(i) determining the sequence of the bead-bound peptide.

"Protein" means any compound which comprises amino acids, including peptides , polypeptides, fusion proteins, etc.

"in conjunction with" means any physical interaction that results in co-localization.

Moreover, for the purposes of the present invention, the term "a" or "an" entity refers to one or more of that entity; for example, "a protein" or "a nucleic acid molecule" refers to one or more of those compounds or at least one compound. As such, the terms "a" (or "an"), "one or more" and "at least one" can be used interchangeably herein. It is also to be noted that the terms "comprising", "including", and "having" can be used interchangeably. Furthermore, a compound "selected from the group consisting of" refers to one or more of the compounds in the list that follows, including mixtures (i.e., combinations) of two or more of the compounds. According to the present invention, an isolated, or biologically pure, protein or nucleic acid molecule is a compound that has been removed from its natural milieu. As such, "isolated" and "biologically pure" do not necessarily reflect the extent to which the compound has been purified. An isolated compound of the present invention can be obtained from its natural source, can be produced using molecular biology techniques or can be produced by chemical synthesis.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. I shows a scheme for screening a random phage library for binding to HA.

FIG. 2 shows binding of two synthetic peptides to a hyaluronic binding domain.

FIG. 3 shows affinities of peptides that bind to the HA binding domain.

FIG. 4 shows a table describing abbreviations of the amino acids.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides HA mimics comprising a polypeptide having alternating acidic / non-acidic residues, wherein said mimic binds to the hyaluronic acid binding domain of the receptor for hyaluronan mediated mobility. Those HA mimics wherein said polypeptide is four to 15 residues in length are preferred. More preferred are those HA mimics as described, wherein said residues include at least one dextrorotatory residue. Most preferred are HA mimics comprising an amino acid sequence selected from the group consisting of: SEQ ID NO 1; SEQ ID NO 2; SEQ ID NO 3; SEQ ID NO 4; SEQ ID NO 5; SEQ ID NO 6; SEQ ID NO 7; SEQ ID NO 8; SEQ ID NO 9; SEQ ID NO 10; SEQ ID NO 11; SEQ ID NO 12; SEQ ID NO 13; SEQ ID NO 14; SEQ ID NO 15; SEQ ID NO 16; SEQ ID NO 17; SEQ ID NO 18; SEQ ID NO 19; and SEQ ID NO 20, or a homologue thereof, wherein said homologue binds to the hyaluronic acid binding domain of the receptor for hyaluronan mediated mobility.

In a preferred embodiment of the present invention, the HA mimics comprising the sequence:

$$X\text{-}A/a\text{-}X\text{-}A/a\text{-}X\text{-}a\text{-}X\text{-}a_1$$

wherein (X) can be any amino acid, (A) is L-glutamate or L-aspartate and (a) and (a_1) are D-glutamate or D-aspartate.

Those wherein X is hydrophobic are more preferred and those wherein X is hydrophobic and a_1 is D-glutamate are most preferred.

Also provided are HA mimics comprising a polypeptide comprising double hydrophobic residues, wherein said mimic binds to the hyaluronic

acid binding domain of the TSG-6. In particular, provided are HA mimics of which are selected from the group consisting of: GYYFNVAM; WAYNFLVM; TQSLNNHM; WWPFINAY; WWKADMVG; WWPFINAY; MALQLPYY; IIYEEFFV; ISINNRWY; VTPPVYFT; QIRNGWFW; SWWFGPLA; GDWEQILT; PAGFGWNL; NMRFNIEN; and QMTFFDGV, or a homologue thereof, wherein said homologue binds to hyaluronic acid binding domain of the TSG-6. Nucleic acids which encode the above compounds are also part of the present invention.

The present invention also provides nucleic acid compounds comprising a nucleic acid which encodes an amino acid sequence selected from the group consisting of: SEQ ID NO 1; SEQ ID NO 2; SEQ ID NO 3; SEQ ID NO 4; SEQ ID NO 5; SEQ ID NO 6; SEQ ID NO 7; SEQ ID NO 8; SEQ ID NO 9; SEQ ID NO 10; SEQ ID NO 11; SEQ ID NO 12; SEQ ID NO 13; SEQ ID NO 14; SEQ ID NO 15; SEQ ID NO 16; SEQ ID NO 17; SEQ ID NO 18; SEQ ID NO 19; and SEQ ID NO 20; and homologues thereof, wherein said homologue binds to the hyaluronic acid binding domain of the receptor for hyaluronan mediated mobility.

Also provided are compositions of matter comprising an HA mimic of the present invention, in particular, those which comprise a non-steroidal anti-inflammatory drug.

The present invention also provides methods to affect a hyaluronidase-mediated biological response in a patient in need of such affecting , comprising administering an HA mimic of the present invention.

The present invention also provides methods to inhibit hyaluronidase activity in a patient in need of such inhibition, comprising administering an HA mimic of the present invention.

The present invention also provides methods to bind receptors that bind hyaluronic acid, comprising introducing an HA mimic of the present invention.

The present invention also provides methods to mimic hyaluronic acid in a biological system, comprising introducing an HA mimic of the present invention.

The present invention also provides methods to affect cell signalling associated with hyaluronic acid/hyaluronic acid receptor interactions, comprising introducing an HA mimic of the present invention.

The present invention also provides methods to treat hyaluronic acid-associated disease in a patient in need of such treatment, comprising administering an HA mimic of the present invention. In particular, those methods wherein said disease is selected from the group consisting of: inflammation; tumor angiogenesis; skin disease; bone disease; wound healing;osteoarthritis; rheumatoid arthritis; infectious disease; immune disease; and cardiovascular disease are preferred.

The present invention also provides methods to inhibit metastasis in a patient in need of such inhibition, comprising administering an HA mimic of the present invention.

The present invention also provides methods to inhibit fertilization in a patient in need of such inhibition, comprising administering an HA mimic of the present invention.

The present invention also provides methods to introduce an agent into a cell which binds hyaluronic acid, comprising administering the agent in conjunction with an HA mimic of the present invention.

The present invention also provides methods to isolate peptides that mimic a ligand's binding to a receptor comprising the steps of:

(a) preparing a random library of peptides and binding said library to a bead;
(b) placing the library in contact with the receptor under conditions for binding;
(c) washing off unbound peptides;
(d) contacting the washed bead-bound library with anti-receptor antibody;
(e) detecting and selecting beads having antibody bound thereto;
(f) eluting the antibody;
(g) repeating steps (a) through (f) and then repeating steps (a) through (c), and then incubating the beads in the presence of receptor prior to adding anti-receptor antibody;
(h) detecting and selecting beads that did not bind antibody;
(i) determining the sequence of the bead-bound peptide.

HA mimic homologs of the present invention can be produced using techniques known in the art including, but not limited to, direct modifications to the peptide or modifications to the gene encoding the peptide using, for example, classic or recombinant nucleic acid techniques to effect random or targeted mutagenesis.

A HA mimic of the present invention, including a homolog, can be identified in a straight-forward manner by the protein's ability to inhibit an HA target enzyme.

One embodiment of a HA mimic of the present invention is a fusion protein that includes a HA receptor binding domain attached to one or more fusion segments. Suitable fusion segments for use with the present invention include, but are not limited to, segments that can: enhance a protein's stability and/or assist purification of an HA mimic (e.g., by affinity chromatography). A suitable fusion segment can be a domain of any size that has the desired function (e.g., imparts increased stability, imparts increased immunogenicity to a protein, and/or simplifies purification of a protein). Fusion proteins are preferably produced by culturing a recombinant cell transformed with a fusion nucleic acid molecule that encodes a protein including the fusion segment attached to either the carboxyl and/or amino terminal end of an HA receptor binding domain. Preferred fusion segments include a metal binding domain (e.g., a poly-histidine segment); an immunoglobulin binding domain (e.g., Protein A; Protein G; T cell; B cell; Fc receptor or complement protein antibody-binding domains); a sugar binding domain (e.g., a maltose binding domain); a "tag" domain (e.g., at least a portion of ß-galactosidase, a strep tag peptide, other domains that can be purified using compounds that bind to the domain, such as monoclonal antibodies); and/or a linker and enzyme domain (e.g., alkaline phosphatase domain connected to A HA mimic by a linker). More preferred fusion segments include metal binding domains, such as a poly-histidine segment; a maltose binding domain; a strep tag peptide; and a phage T7 S10 peptide.

An HA mimic of the present invention can also be a chimeric molecule comprising an HA mimic and a second molecule. In particular,

there is provided a chimeric molecule that enables the chimeric molecule to be bound to a surface in such a manner that the chimera inhibits an HA-target enzyme in essentially the same manner as an HA mimic that is not bound to a surface. An example of a suitable second molecule includes a portion of an immunoglobulin molecule or another ligand that has a suitable binding partner that can be immobilized on a substrate, e.g., biotin and avidin, or a metal-binding protein and a metal (e.g., His), or a sugar-binding protein and a sugar (e.g., maltose).

Nucleic acid molecules comprising a nucleic acid molecule which encodes the present HA mimics are also provided by the present invention. In particular, there are provided nucleic acids encoding the present HA mimics, wherein said HA mimics comprise, an amino acid sequence selected the group consisting of: SEQ ID NO 1; SEQ ID NO 2; SEQ ID NO 3; SEQ ID NO 4; SEQ ID NO 5; SEQ ID NO 6; SEQ ID NO 7; SEQ ID NO 8; SEQ ID NO 9; SEQ ID NO 10; SEQ ID NO 11; SEQ ID NO 12; SEQ ID NO 13; SEQ ID NO 14; SEQ ID NO 15; SEQ ID NO 16; SEQ ID NO 17; SEQ ID NO 18; SEQ ID NO 19; and SEQ ID NO 20.

The present invention also comprises expression vectors and recombinant cells comprising the present nucleic acid molecules. Also provided are fusion constructs using the present nucleic acid compounds.

One approach is to create fusogenic peptides, in which a short (18-mer) peptide is attached to the effector peptide that causes endocytotic uptake of the peptide into the cell. J. Hawiger, Noninvasive intracellular delivery of functional peptides and proteins, Curr. Opinion Chemical Biology 3, 89-94 (1999) and A. Prochiantz, Getting hydrophilic compounds into cells: lessons from heomeopeptides. Cur. Opin. Neurobiol 6, 629-630 (1996)

Included within the scope of the present invention, with particular regard to the nucleic acids above, are degenerate sequences and homologues. The present invention also includes variants due to laboratory manipulation, such as, but not limited to, variants produced during polymerase chain reaction amplification or site-directed mutagenesis. It is also well known that there is a substantial amount of redundancy in the various codons which code for specific amino acids. Therefore, this invention is also directed to those nucleic acid sequences which contain alternative codons which code for the eventual translation of the identical amino acid. Also included within the scope of this invention are mutations either in the nucleic acid sequence or the translated protein which do not substantially alter the ultimate physical properties of the expressed protein. For example, substitution of valine for leucine, arginine for lysine, or asparagine for glutamine may not cause a change in functionality of the polypeptide.

Knowing the nucleic acid sequences of certain HA mimic nucleic acid molecules of the present invention allows one skilled in the art to, for example, (a) make copies of those nucleic acid molecules, (b) obtain nucleic acid molecules including at least a portion of such nucleic acid molecules (e.g., nucleic acid molecules including full-length genes, full-length coding regions, regulatory control sequences, truncated coding regions), and (c) obtain HA mimic nucleic acid molecules. Such nucleic acid molecules can be obtained in a variety of ways including screening appropriate expression libraries with antibodies of the present invention; traditional cloning techniques using oligonucleotide probes of the present invention to screen appropriate libraries of DNA; and PCR amplification of appropriate libraries or DNA using oligonucleotide primers of the present invention. Preferred libraries to screen or from which to amplify nucleic acid molecules include livestock (cattle, horse, pig) and companion animal (dog and cat) cDNA libraries as well as genomic DNA libraries. Similarly, preferred DNA sources

to screen or from which to amplify nucleic acid molecules include adult cDNA and genomic DNA. Techniques to clone and amplify genes are disclosed, for example, in Sambrook et al., *ibid.*

One embodiment of the present invention includes a recombinant vector, which includes at least one isolated nucleic acid molecule of the present invention, inserted into any vector capable of delivering the nucleic acid molecule into a host cell. Such a vector contains heterologous nucleic acid sequences, that is, nucleic acid sequences that are not naturally found adjacent to nucleic acid molecules of the present invention and that preferably are derived from a species other than the species from which the nucleic acid molecule(s) are derived. The vector can be either RNA or DNA, either prokaryotic or eukaryotic, and typically is a virus or a plasmid. Recombinant vectors can be used in the cloning, sequencing, and/or otherwise manipulation of the HA mimic nucleic acid molecules of the present invention.

One type of recombinant vector, referred to herein as a recombinant molecule, comprises a nucleic acid molecule of the present invention operatively linked to an expression vector. The phrase "operatively linked" refers to insertion of a nucleic acid molecule into an expression vector in a manner such that the molecule is able to be expressed when transformed into a host cell. As used herein, an expression vector is a DNA or RNA vector that is capable of transforming a host cell and of effecting expression of a specified nucleic acid molecule. Preferably, the expression vector is also capable of replicating within the host cell. Expression vectors can be either prokaryotic or eukaryotic, and are typically viruses or plasmids. Expression vectors of the present invention include any vectors that function (i.e., direct gene expression) in recombinant cells of the present invention, including in bacterial, fungal, endoparasite, insect, other animal, and plant cells. Preferred

expression vectors of the present invention can direct gene expression in bacterial, yeast, insect and mammalian cells and more preferably in the cell types disclosed herein.

In particular, expression vectors of the present invention contain regulatory sequences such as transcription control sequences, translation control sequences, origins of replication, and other regulatory sequences that are compatible with the recombinant cell and that control the expression of nucleic acid molecules of the present invention. In particular, recombinant molecules of the present invention include transcription control sequences. Transcription control sequences are sequences which control the initiation, elongation, and termination of transcription. Particularly important transcription control sequences are those which control transcription initiation, such as promoter, enhancer, operator and repressor sequences. Suitable transcription control sequences include any transcription control sequence that can function in at least one of the recombinant cells of the present invention. A variety of such transcription control sequences are known to those skilled in the art. Preferred transcription control sequences include those which function in bacterial, yeast, insect and mammalian cells, such as, but not limited to, *tac*, *lac*, *trp*, *trc*, oxy-pro, omp/lpp, rrnB, bacteriophage lambda (such as lambda p_L and lambda p_R and fusions that include such promoters), bacteriophage T7, T7*lac*, bacteriophage T3, bacteriophage SP6, bacteriophage SP01, metallothionein, alpha-mating factor, *Pichia* alcohol oxidase, alphavirus subgenomic promoters (such as Sindbis virus subgenomic promoters), antibiotic resistance gene, baculovirus, *Heliothis zea* insect virus, vaccinia virus, herpesvirus, raccoon poxvirus, other poxvirus, adenovirus, cytomegalovirus (such as intermediate early promoters), simian virus 40, retrovirus, actin, retroviral long terminal repeat, Rous sarcoma virus, heat shock, phosphate and nitrate transcription control sequences as well as other sequences capable of controlling gene expression in

prokaryotic or eukaryotic cells. Additional suitable transcription control sequences include tissue-specific promoters and enhancers as well as lymphokine-inducible promoters (e.g., promoters inducible by interferons or interleukins). Transcription control sequences of the present invention can also include naturally-occurring transcription control sequences naturally associated with humans. The present invention also comprises expression vectors comprising a nucleic acid molecule described herein.

Recombinant DNA technologies can be used to improve expression of transformed nucleic acid molecules by manipulating, for example, the number of copies of the nucleic acid molecules within a host cell, the efficiency with which those nucleic acid molecules are transcribed, the efficiency with which the resultant transcripts are translated, and the efficiency of post-translational modifications. Recombinant techniques useful for increasing the expression of nucleic acid molecules of the present invention include, but are not limited to, operatively linking nucleic acid molecules to high-copy number plasmids, integration of the nucleic acid molecules into one or more host cell chromosomes, addition of vector stability sequences to plasmids, substitutions or modifications of transcription control signals (e.g., promoters, operators, enhancers), substitutions or modifications of translational control signals (e.g., ribosome binding sites, Shine-Dalgarno sequences), modification of nucleic acid molecules of the present invention to correspond to the codon usage of the host cell, deletion of sequences that destabilize transcripts, and use of control signals that temporally separate recombinant cell growth from recombinant enzyme production during fermentation. The activity of an expressed recombinant protein of the present invention may be improved by fragmenting, modifying, or derivatizing nucleic acid molecules encoding such a protein.

Also provided by the present invention are recombinant cells transformed with a nucleic acid described herein.

Transformation of a nucleic acid molecule into a cell can be accomplished by any method by which a nucleic acid molecule can be inserted into the cell. Transformation techniques include, but are not limited to, transfection, electroporation, microinjection, lipofection, adsorption, and protoplast fusion. A recombinant cell may remain unicellular or may grow into a tissue, organ or a multicellular organism. Transformed nucleic acid molecules of the present invention can remain extrachromosomal or can integrate into one or more sites within a chromosome of the transformed (i.e., recombinant) cell in such a manner that their ability to be expressed is retained.

Suitable host cells to transform include any cell that can be transformed with a nucleic acid molecule of the present invention. Host cells can be either untransformed cells or cells that are already transformed with at least one nucleic acid molecule (e.g., nucleic acid molecules encoding one or more proteins of the present invention and/or other proteins useful in the production of multivalent vaccines). Host cells of the present invention either can be endogenously (i.e., naturally) capable of producing HA mimic of the present invention or can be capable of producing such proteins after being transformed with at least one nucleic acid molecule of the present invention. Host cells of the present invention can be any cell capable of producing at least one protein of the present invention, and include bacterial, fungal (including yeast), other insect, other animal and plant cells. Preferred host cells include bacterial, mycobacterial, yeast, parasite, insect and mammalian cells. More preferred host cells include *Salmonella, Escherichia, Bacillus, Listeria, Saccharomyces, Spodoptera, Mycobacteria, Trichoplusia,* BHK (baby hamster kidney) cells, COS (e.g., COS-7) cells, and Vero cells. Particularly preferred

host cells are *Escherichia coli*, including *E. coli* K-12 derivatives; and insect cell systems which utilize baculovirus.

A recombinant cell is preferably produced by transforming a host cell with one or more recombinant molecules, each comprising one or more nucleic acid molecules of the present invention operatively linked to an expression vector containing one or more transcription control sequences. The phrase "operatively linked" refers to insertion of a nucleic acid molecule into an expression vector in a manner such that the molecule is able to be expressed when transformed into a host cell.

A recombinant cell of the present invention includes any cell transformed with at least one of any nucleic acid molecule of the present invention. Suitable and preferred nucleic acid molecules as well as suitable and preferred recombinant molecules with which to transform cells are disclosed herein.

The translation of the RNA into a peptide or a protein will result in the production of the HA mimic protein which can be identified, for example, by the activity of HA mimic or by immunological reactivity with an anti-HA mimic antibody. In this method, pools of mRNA isolated from HA mimic-producing cells can be analyzed for the presence of an RNA which encodes at least a portion of the HA mimic protein. Further fractionation of the RNA pool can be done to purify the HA mimic RNA from non-HA mimic RNA. The peptide or protein produced by this method may be analyzed to provide amino acid sequences which in turn are used to provide primers for production of HA mimic cDNA, or the RNA used for translation can be analyzed to provide nucleotide sequences encoding HA mimic and produce probes for the production of HA mimic cDNA. These methods are known in the art and can be found in, for example, Sambrook, J., Fritsch, E. F., Maniatis,

T. in *Molecular Cloning: A Laboratory Manual, Second Edition*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. 1989.

Other types of libraries, as well as libraries constructed from other cells or cell types, may be useful for isolating HA mimic-encoding DNA. Other types of libraries include, but are not limited to, cDNA libraries derived from other mammals or cell lines derived from other mammals, and genomic DNA libraries. Preparation of cDNA libraries can be performed by standard techniques. Well known cDNA library construction techniques can be found in, for example, Sambrook, J., et al., *ibid.*

In one embodiment, an isolated protein of the present invention is produced by culturing a cell capable of expressing the protein under conditions effective to produce the protein, and recovering the protein. A preferred cell to culture is a recombinant cell of the present invention. Effective culture conditions include, but are not limited to, effective media, bioreactor, temperature, pH and oxygen conditions that permit protein production. An effective medium refers to any medium in which a cell is cultured to produce a HA mimic of the present invention. Such a medium typically comprises an aqueous medium having assimilable carbon, nitrogen and phosphate sources, and appropriate salts, minerals, metals and other nutrients, such as vitamins. Cells of the present invention can be cultured in conventional fermentation bioreactors, shake flasks, test tubes, microtiter dishes, and petri plates. Culturing can be carried out at a temperature, pH and oxygen content appropriate for a recombinant cell. Such culturing conditions are within the expertise of one of ordinary skill in the art.

Depending on the vector and host system used for production, resultant proteins of the present invention may either remain within the recombinant cell; be secreted into the fermentation medium; be secreted into

a space between two cellular membranes, such as the periplasmic space in *E. coli*; or be retained on the outer surface of a cell or viral membrane. The phrase "recovering the protein", as well as similar phrases, refers to collecting the whole fermentation medium containing the protein and need not imply additional steps of separation or purification. Proteins of the present invention can be purified using a variety of standard protein purification techniques, such as, but not limited to, affinity chromatography, ion exchange chromatography, filtration, electrophoresis, hydrophobic interaction chromatography, gel filtration chromatography, reverse phase chromatography, concanavalin A chromatography, chromatofocusing and differential solubilization. Proteins of the present invention are preferably retrieved in "substantially pure" form. As used herein, "substantially pure" refers to a purity that allows for the effective use of the protein as a therapeutic composition or diagnostic. A therapeutic composition for animals, for example, should exhibit few impurities.

In addition, a recombinant HA mimic can be separated from other cellular proteins by use of an immunoaffinity column made with monoclonal or polyclonal antibodies specific for the HA mimic, or polypeptide fragments of the HA mimic.

The present invention also includes isolated (i.e., removed from their natural milieu) antibodies that selectively bind to an HA mimic protein of the present invention or a mimetope thereof (i.e., anti-HA mimic antibodies). As used herein, the term "selectively binds to" the HA mimic protein refers to the ability of antibodies of the present invention to preferentially bind to specified proteins and mimetopes thereof of the present invention. Binding can be measured using a variety of methods standard in the art including enzyme immunoassays (e.g., ELISA), immunoblot assays, etc.; see, for example, Sambrook et al., *ibid*. An anti-HA mimic antibody preferably

selectively binds to A HA mimic in such a way as to reduce the activity of that protein. These antibodies may be admixed or conjugated with additional materials, such as cytotic agents or other antibody fragments.

Isolated antibodies of the present invention can include antibodies in a bodily fluid (such as, but not limited to, serum), or antibodies that have been purified to varying degrees. Antibodies of the present invention can be polyclonal or monoclonal. Functional equivalents of such antibodies, such as antibody fragments and genetically-engineered antibodies (including single chain antibodies or chimeric antibodies that can bind to more than one epitope) are also included in the present invention.

A preferred method to produce antibodies of the present invention includes (a) administering to an animal an effective amount of a protein, peptide or mimetope thereof of the present invention to produce the antibodies and (b) recovering the antibodies. In another method, antibodies of the present invention are produced recombinantly using techniques as heretofore disclosed to produce HA mimic proteins of the present invention.

Compositions of the present invention can be administered to any animal having at least one HA mimic-target enzyme that can be inhibited by a therapeutic compound of the present invention or by a protein expressed by a nucleic acid molecule contained in a therapeutic composition. Preferred animals to treat are humans, although other mammals, such as cattle, pigs, sheep, horses, cats, dogs, and other pets, work and/or economic food animals are also within the scope of the present invention.

Therapeutic compositions of the present invention can be formulated in an excipient that the animal to be treated can tolerate. Examples of such excipients include water, saline, Ringer's solution, dextrose solution, Hank's

solution, and other aqueous physiologically balanced salt solutions. Nonaqueous vehicles, such as fixed oils, sesame oil, ethyl oleate, or triglycerides may also be used. Other useful formulations include suspensions containing viscosity enhancing agents, such as sodium carboxymethylcellulose, sorbitol, or dextran. Excipients can also contain minor amounts of additives, such as substances that enhance isotonicity and chemical stability. Examples of buffers include phosphate buffer, bicarbonate buffer and Tris buffer, while examples of preservatives include thimerosal, cresols, formalin and benzyl alcohol. Standard formulations can either be liquid injectables or solids which can be taken up in a suitable liquid as a suspension or solution for injection. Thus, in a non-liquid formulation, the excipient can comprise dextrose, human serum albumin, preservatives, etc., to which sterile water or saline can be added prior to administration.

Administration of the present compounds can be by a variety of routes known to those skilled in the art including, but not limited to, subcutaneous, intradermal, intravenous, intranasal, oral, transdermal, intramuscular routes and other parenteral routes.

In one embodiment of the present invention, a therapeutic composition can include an adjuvant. A preferred adjuvant is a member of the group of non-steroidal antiinflammatory drugs, such as ibuprofen.

In another embodiment of the present invention, a therapeutic composition can include a carrier. Carriers include compounds that increase the half-life of a therapeutic composition in the treated animal. Suitable carriers include, but are not limited to, polymeric controlled release vehicles, biodegradable implants, liposomes, bacteria, viruses, other cells, oils, esters, and glycols.

Another embodiment of the present invention is a controlled release formulation that is capable of slowly releasing a composition of the present invention into an animal. As used herein, a controlled release formulation comprises a composition of the present invention in a controlled release vehicle. Suitable controlled release vehicles include, but are not limited to, biocompatible polymers, other polymeric matrices, capsules, microcapsules, microparticles, bolus preparations, osmotic pumps, diffusion devices, liposomes, lipospheres, and transdermal delivery systems. Other controlled release formulations of the present invention include liquids that, upon administration to an animal, form a solid or a gel *in situ*. Preferred controlled release formulations are biodegradable (i.e., bioerodible).

A preferred controlled release formulation of the present invention is capable of releasing a composition of the present invention into the blood of an animal at a constant rate sufficient to attain therapeutic dose levels of the composition to reduce HA-mediated biological responses in the animal. The therapeutic composition is preferably released over a period of time ranging from about 1 day to about 12 months, and include release over a 2, 3, 4 , 5, 6, 7 day through a 30 day time period.

Acceptable protocols to administer therapeutic compositions of the present invention in an effective manner include individual dose size, number of doses, frequency of dose administration, and mode of administration. Determination of such protocols can be accomplished by those skilled in the art. A suitable single dose is a dose that is capable of protecting (i.e., preventing or treating) an animal from disease when administered one or more times over a suitable time period. The need for additional administrations of a therapeutic composition can be determined by one of skill in the art in accordance with the given condition of a patient.

According to one embodiment, a nucleic acid molecule of the present invention can be administered to an animal in a fashion to enable expression of that nucleic acid molecule into an HA mimic protein in the animal. Nucleic acid molecules can be delivered to an animal in a variety of methods including, but not limited to, (a) administering a naked (i.e., not packaged in a viral coat or cellular membrane) nucleic acid molecule (e.g., as naked DNA molecules, such as is taught, for example in Wolff et al., 1990, *Science* 247, 1465-1468) or (b) administering a nucleic acid molecule packaged as a recombinant virus or as a recombinant cell (i.e., the nucleic acid molecule is delivered by a viral or cellular vehicle).

A naked nucleic acid molecule of the present invention includes a nucleic acid molecule of the present invention and preferably includes a recombinant molecule of the present invention that preferably is replication, or otherwise amplification, competent. A naked nucleic acid of the present invention can comprise one or more nucleic acid molecules of the present invention in the form of, for example, a bicistronic recombinant molecule having, for example one or more internal ribosome entry sites. Preferred naked nucleic acid molecules include at least a portion of a viral genome (i.e., a viral vector). Preferred viral vectors include those based on alphaviruses, poxviruses, adenoviruses, herpesviruses, picornaviruses, and retroviruses, with those based on alphaviruses (such as Sindbis or Semliki virus), species-specific herpesviruses and species-specific poxviruses being particularly preferred. Any suitable transcription control sequence can be used, including those disclosed as suitable for protein production. Particularly preferred transcription control sequence include cytomegalovirus intermediate early (preferably in conjunction with Intron-A), Rous Sarcoma Virus long terminal repeat, and tissue-specific transcription control sequences, as well as transcription control sequences endogenous to viral vectors if viral vectors are used. The incorporation of "strong" poly(A) sequences are also preferred.

Naked nucleic acid molecules of the present invention can be administered by a variety of methods. Suitable delivery methods include, for example, intramuscular injection, subcutaneous injection, intradermal injection, intradermal scarification, particle bombardment, oral application, and nasal application, with intramuscular injection, intradermal injection, intradermal scarification and particle bombardment being preferred. A preferred single dose of a naked DNA molecule ranges from about 1 nanogram (ng) to about 1 milligram (mg), depending on the route of administration and/or method of delivery, as can be determined by those skilled in the art. Examples of administration methods are disclosed, for example, in U.S. Patent No. 5,204,253, by Bruner, et al., issued April 20, 1993, PCT Publication No. W0 95/19799, published July 27, 1995, by McCabe, and PCT Publication No. WO 95/05853, published March 2, 1995, by Carson, et al. Naked DNA molecules of the present invention can be contained in an aqueous excipient (e.g., phosphate buffered saline) and/or with a carrier (e.g., lipid-based vehicles), or it can be bound to microparticles (e.g., gold particles).

A recombinant virus of the present invention includes a recombinant molecule of the present invention that is packaged in a viral coat and that can be expressed in an animal after administration. Preferably, the recombinant molecule is packaging-deficient and/or encodes an attenuated virus. A number of recombinant viruses can be used, including, but not limited to, those based on alphaviruses, poxviruses, adenoviruses, herpesviruses, picornaviruses and retroviruses.

When administered to an animal, a recombinant virus of the present invention infects cells within the recipient animal and directs the production of a protein molecule that is capable of affecting HA-mediated biological responses in the animal. For example, a recombinant virus comprising an

HA mimic nucleic acid molecule of the present invention is administered according to a protocol that results in the animal producing an amount of protein sufficient to affect HA-mediated biological responses. Administration protocols are similar to those described herein for protein-based compositions, with subcutaneous, intramuscular, intranasal and oral administration routes being preferred.

Pharmaceutically useful compositions comprising an HA mimic DNA or an HA mimic protein, may be formulated according to known methods such as by the admixture of a pharmaceutically acceptable carrier, or by modification with additional chemical moieties so as to form a chemical derivative. Examples of such carriers, modifications and methods of formulation may be found in Remington's Pharmaceutical Sciences. To form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain an effective amount of the protein or DNA.

The present invention also has the objective of providing suitable topical, oral, systemic and parenteral formulations of the pharmaceutical compounds herein provided. The formulations can be administered in a wide variety of therapeutic dosage forms in conventional vehicles for administration. For example, the compounds can be formulated for oral administration in the form of tablets, capsules (each including timed release and sustained release formulations), pills, powders, granules, elixirs, tinctures, solutions, suspensions, syrups and emulsions, or by injection. Likewise, they may also be administered intravenously (both bolus and infusion), during angioplasty/catheterization, intraperitoneally, subcutaneously, topically with or without occlusion, or intramuscularly, all using forms well known to those of ordinary skill in the pharmaceutical arts.

An HA mimic of the present invention can be combined with a buffer in which the HA mimic molecule is solubilized, and/or with a carrier. Suitable buffers and carriers are known to those skilled in the art. Examples of suitable buffers include any buffer in which an HA mimic can function to inhibit its target enzyme(s), such as, but not limited to, phosphate buffered saline, water, saline, phosphate buffer, bicarbonate buffer, HEPES buffer (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffered saline), TES buffer (Tris-EDTA buffered saline), Tris buffer and TAE buffer (Tris-acetate-EDTA). Examples of carriers include, but are not limited to, polymeric matrices, toxoids, and serum albumins, such as bovine serum albumin.

In the methods of the present invention, the compounds herein described in detail can form the active ingredient, and are typically administered in admixture with suitable pharmaceutical diluents, excipients or carriers (collectively referred to herein as "carrier" materials) suitably selected with respect to the intended form of administration, that is, oral tablets, capsules, elixirs, syrups and the like, and consistent with conventional pharmaceutical practices.

For instance, for oral administration in the form of a tablet or capsule, the active drug component can be combined with an oral, non-toxic pharmaceutically acceptable inert carrier such as ethanol, glycerol, water, and the like. Moreover, when desired or necessary, suitable binders, lubricants, disintegrating agents and coloring agents can also be incorporated into the mixture. Suitable binders include, without limitation, starch, gelatin, natural sugars such as glucose or beta-lactose, corn sweeteners, natural and synthetic gums such as acacia, tragacanth or sodium alginate, carboxymethylcellulose, polyethylene glycol, waxes and the like. Lubricants used in these dosage forms include, without limitation, sodium oleate, sodium stearate, magnesium stearate, sodium benzoate, sodium acetate, sodium chloride, and

the like. Disintegrators include, without limitation, starch, methyl cellulose, agar, bentonite, xanthan gum and the like.

For liquid forms the active drug component can be combined in suitably flavored suspending or dispersing agents such as the synthetic and natural gums, for example, tragacanth, acacia, methylcellulose and the like. Other dispersing agents which may be employed include glycerin and the like. For parenteral administration, sterile suspensions and solutions are desired. Isotonic preparations which generally contain suitable preservatives are employed when intravenous administration is desired.

Topical preparations containing the active drug component can be admixed with a variety of carrier materials well known in the art, such as, e.g., alcohols, aloe vera gel, allantoin, glycerine, vitamin A and E oils, mineral oil, PPG2 myristyl propionate, and the like, to form, e.g., alcoholic solutions, topical cleansers, cleansing creams, skin gels, skin lotions formulations. The compounds of the present invention can also be administered in the form of liposome delivery systems, such as small unilamellar vesicles, large unilamellar vesicles and multilamellar vesicles. Liposomes can be formed from a variety of phospholipids, such as cholesterol, stearylamine or phosphatidylcholines.

The compounds of the present invention may also be coupled with soluble polymers as targetable drug carriers. Such polymers can include polyvinyl-pyrrolidone, pyran copolymer, polyhydroxypropylmethacryl-amidephenol, polyhydroxy-ethylaspartamidephenol, or polyethyleneoxidepolylysine substituted with palmitoyl residues. Furthermore, the compounds of the present invention may be coupled to a class of biodegradable polymers useful in achieving controlled release of a drug, for example, polylactic acid, polyepsilon caprolactone, polyhydroxy

butyric acid, polyorthoesters, polyacetals, polydihydro-pyrans, polycyanoacrylates and cross-linked or amphipathic block copolymers of hydrogels.

Therefore, methods are also provided herein, which utilize the compounds, formulations, compositions and protocols described above. In particular, there are provided methods to antagonize or inhibit HA-target enzymes, comprising administering an HA mimic of the present invention. Preferred methods utilize the preferred and most preferred HA mimics.

The present invention also provides methods to treat and/or reduce the risk of HA-related diseases in a patient in need of such treatment, comprising administering the presently-disclosed HA mimics.

Lastly, the present invention also provides methods for producing the present HA mimics in bodily fluid, comprising: producing a transgenic animal that expresses in bodily fluid a transgene which encodes an HA mimic of the present invention, wherein the HA mimic is secreted into the bodily fluid produced by the transgenic animal; collecting bodily fluid from the transgenic animal, which bodily fluid contains the HA mimic; and isolating the HA mimic from the collected bodily fluid. Preferred are methods wherein the bodily fluid is selected from the group consisting of: milk or urine. Those methods wherein the bodily fluid is milk and the animal is selected from the group consisting of: goat; sheep; and cow are more preferred. Most preferred are methods for producing an HA mimic in goat milk, comprising: producing a transgenic goat that expresses in mammary tissue a transgene which encodes an HA mimic of the present invention, wherein the HA mimic is secreted into the milk produced by the transgenic goat; collecting milk from the transgenic goat which milk contains the HA mimic; and isolating the HA mimic from the collected milk. This aspect of the invention can be

accomplished according to US Patent Serial Number 5,843,705, which patent is hereby incorporated by reference in its entirety.

The present invention also provides methods to identify the ability of a test compound to interfere with the present HA mimic/target enzyme interaction, comprising: contacting the test compound with a protein of the present invention; and determining whether the test compound and said protein interact.

The following examples illustrate the present invention without, however, limiting it. It is to be noted that the Examples include a number of molecular biology, microbiology, immunology and biochemistry techniques considered to be known to those skilled in the art. Disclosure of such techniques can be found, for example, in Sambrook et al., *ibid.*, and related references.

Examples

Example 1 Screening of Phage Library

A completely random phage library expressing a 15-amino acid peptide was used to screen phage that can bind to the HA binding domains. The principle of the screening process relies on selectively eluting phage that bind to the HA binding domains. Three constructs expressing the HA binding domains of RHAMM (Receptor of HA Mediated Motility) were prepared. The first two GST-P1 (70 amino acids) and GST-P6 (68 amino acids are glutathione-S-transferase fusion constructs. The third is a thioredoxin fusion construct (62 amino acids 312c). The HA binding domain portions of these polypeptides are identical except for several amino acids on the N or C terminus. A

portion of the phage screens were performed with 312c as the target peptide while all binding assays used GST-P1. All constructs have a thrombin cleavage site, which was key to the selective elution method used.

To screen phage which selectively bound to the HA binding domains, the following method was used. Purified GST-P6 or 312c (0.1mg/mL) were immobilized on a 30 mm polystyrene petri dish in 50 mM Tris, pH 8.0. The phage library was applied in binding buffer (Tris buffered saline pH 7.5, 0.05% Tween, 0.1% polyvinylpyrolidone) (TBST-PVP). Nonspecific phage were washed off,and the phage bound to the HA binding domains were recovered either by cleaving the HA binding domain from the fusion protein using thrombin (first and second rounds of screening), or eluting with HA (third round of screening), as summarized in FIG. 1. Finally, the peptide encoding DNAs present in the phages were sequenced. Table 1 shows the numbers of phages recovered during the second round of screening.

Table 1

Screen Step	cfU/mL		
	312c	GST-P6	Control
Input	1×10^{10}	1×10^{10}	1×10^{10}
First Wash	7×10^{4}	6×10^{4}	6×10^{4}
Last Wash	8	10	3
40µg/ml HA elution	1160	1320	-
1 mg/mL HA elution	280	160	-
Thrombin cleavage	128	168	20

From the results shown in Table 1, it was concluded that most or all nonspecific phage were washed off. After adding HA, some specific phage were replaced by HA. Therefore, the phage from the HA elution were

considered to have specifically bound to the HA binding domain. The amino acid sequences of eleven peptides (HA1 -HA11; SEQ ID NO: 1 through SEQ ID NO: 11) were determined. Peptides corresponding to HA2-HA5 were synthesized with an amino terminal biotin. The ability of these peptides to bind competitively to GST-P1 was tested. First, GST-P1 was immobilized onto a 96 well plate as described above. After rigorous washing with binding buffer, the biotinylated peptides were applied. Each experiment was performed in quadruplicate. The concentration of peptide applied was titrated such that binding curves could be calculated. The plate was agitated at room temperature for two hours and then washed with binding buffer. Streptavidin alkaline phosphatase (Pharmacia Biotec) was applied in binding buffer at a 1:5000 dilution. This was followed by washing and application of commercially available BCIP/NBT substrate (Sigma). Results are shown in FIG. 2, which shows specific, compatible binding of HA2 and HA3 with GST-P1 L.

Example 2 Screening of One-bead, One-peptide Library

Two bead libraries were constructed in this lab with eight amino acid peptides covalently attached to 100μM Tentagel S (Rapp Polymere GmbH). This method ensures that only one species of peptide is attached to one bead, generally 50nmoles of peptide/bead. One of the bead libraries was designed to be an anionic biased library of 8-mers was designed and prepared on the basis of alternating amino acids with carboxylic acid chains that mimic the 6-carboxylic acids of glucuronic acid (XNXNXNXN; X= any amino acid except Cys, Arg, Lys; N= negatively charged side chains, D-Asp, L-Asp, D-Glu, L-Glu). Both libraries were screened against GST-P1. the method is described as follows:

About 2 ml of beads (approx. 10^5 beads), were prepared in a 5-ml fritted column by sequentially washing and equilibrating in binding buffer. Pure GST-P1 was added to a final concentration of 0.5 ng/ml protein. The beads were incubated with shaking at room temperature for two hours. After vigorous washing in binding buffer, anti-GST-P1 antibody was added (1:2000), the beads were incubated and washed. This was followed by a secondary antibody anti-IgG conjugated to alkaline phosphatase (1:3000). The beads were then washed and placed in a large petri dish. The dish was drained and alkaline phosphatase substrate was added. The substrate was prepared by suspending 50 mg of 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) in 1 ml of DMF and adding 33 µl of this mixture to 10 ml of reaction buffer (TBS pH 8.5 + 1mM $MgCl_2$) Positive beads in reaction buffer would begin to turn blue/purple after 15 minutes. Positive beads were removed using a pipettor and treated with 6 M guanidium hydrochloride, followed by a series of washes in DMF. This screening process was repeated twice, after which approximately 300 candidate beads remained. A third screen was similar to the process mentioned above except that the beads were preincubated with excess natural ligand, in this case 1 mg/mL of partially degraded HA. The beads then collected were those that did not turn blue/purple, indicating that they bound to the same place as the natural ligand. At least 7 candidates were discovered through this screening process that have unique, but related amino acid sequences. The first such positive bead sequences as the primary structure Phe-Asp-Phe Asp-Ser-Glu-Tyr-Glu (SEQ ID NO: 12).

Example 3 Peptide Binding Assays

To verify that the peptides obtained from phage screening indeed bound to the HA binding domains of RHAMM, a solid phase 96-well plate assay was employed. 50 µL GST-P1 (0.25 mg/mL) in glutathione elution buffer was

immobilized in wells of a polystyrene 96-well plate (Greiner). The plate is then blocked with 250 µL TBS-T-PVP-40 bovine serum albumin (BSA) (20 mM Tris, pH 7.5, 130 mM NaCl, 0.1% polyvinylpyrolidone-40, 1% BSA) for 2 hr to overnight with shaking. The plate is washed with TBS-T-PVP-40 (no BSA) and if blocking is required, the plate is incubated with excess HA or chondroitin sulfate (CS) for 1 hr prior to the addition of biotinylated ligand. The biotinylated ligand is incubated for 25 min (determined from an initial timecourse). The plate is washed 3 X, and blocked with TBS-T-PVP-40-BSA for 1 hr. Streptavidin HRP is added at a dilution of 0.5 µg /mL in TBS-T-PVP-40. HA is added to the streptavidin solution, since it is known that streptavidin HRP binds nonspecifically to HA and CS, which inherently causes misleading results in the competition experiments. After 40 min incubation, the plate is washed, and the presence of biotinylated peptide is determined using TMB. Colorimetric development is read at 590 nm (Perkin Elmer HTS 7000). All measurements are performed in quadruplicate. The figure on the following page outlines this method.

For the purposes of generating binding data, the color generated from streptavidin HRP is calibrated and used to estimate the total biotinylated molecules immobilized on the plate. Eadie Hofstee analyses were to calculate binding constants.

Example 4 Pepspots assay

Since the biased library contains both D or L isomers of the alternating charged residues it was necessary to decipher which set of natural or unnatural amino acids provided the best binding to GST-RHAMM-P1. For each peptide obtained from sequencing there are 16 possible combinations (44). This peptide library was constructed as an array of spots on cellulose membrane (Pepspots membranes, Jerini Biotools) where each spot contains a

single species of peptide (3-5nmoles/spot). This membrane contained a total of 68 spots representing 6-18, 6-19, 6-20, 6-21 and 4 control spots taken from a negative bead.

To detect binding we incubated the blocked membrane with GST-RHAMM-P1. After washing, the bound protein was semi-dry transferred from the cellulose membrane onto a PVDF membrane. The PVDF membrane was blocked and incubated with anti-GST, followed by anti-goat antibody-horseradish peroxidase conjugated. To detect bound material, a chemilumiscence detection kit was used followed by exposure to film. The developed film was digitized and quantified to evaluate the strength of GST-RHAMM-P1 binding to each well. To verify the results the experiment was repeated several times at various GST-RHAMM-P1 concentrations.

Example 5 Preferred Binding motifs

Depicted below is a summary of the results found from phage and bead screening experiments. The X stands for unknown amino acids that arose from sequencing difficulties.

Random phage libraries

HA1 WPVSLTVCSAVWCPL (SEQ ID NO 1)
HA2 GVCNADFCWLPAVVV (SEQ ID NO 2)
HA3 SASPSASKLSLMSTV (SEQ ID NO 3)
HA4 IPPILPAYTLLGHPR (SEQ ID NO 4)
HA5 YSVYLSVAHNFVLPS (SEQ ID NO 5)
HA6 HWCLPLLACDTFARA (SEQ ID NO 6)

Biased bead peptides
6-18 MDYEPEQE (SEQ ID NO 7)
6-19 YDSEYESE (SEQ ID NO 8)
6-20 FDFDSEYE (SEQ ID NO 9)
6-21 EDQEAXEX (SEQ ID NO 10)
6-22 EDAENXDX (SEQ ID NO 11)

Random bead peptides
R-1 SGRPYKPP (SEQ ID NO 12)
R-2 YXSSNKPG (SEQ ID NO 13)
R-3 EGEWPVYP (SEQ ID NO 14)
R-4 WNYTEAKG (SEQ ID NO 15)

Figure 1 shows the pertinent results from such an experiment.

The phage results show a motif: ILPA (SEQ ID NO 16) or WLPA (SEQ ID NO 17) with the potential for a hydrophobic amino acid to further govern binding. The presence of prolines is enforced by the random bead libraries where motifs as WPVYP (SEQ ID NO 18) and YKPP (SEQ ID NO 19) are seen. It is believed that a four amino acid sequence in the proper conformation is sufficient to regulate binding to the HA binding domains of RHAMM. The biased library showed that hydrophobic amino acids are important in predisposing a peptide to specific binding: YDSEYESE (SEQ ID NO 20). This information was capitalized on by the Pepspots assay exemplified in Example 4.

The results can be summarized in the following way: In all cases unnatural amino acids facilitated binding. In all but one case the binding of completely natural amino acids provided no binding. Further, we note that a C-terminal D-glutamate acid provides optimal binding and the presence of D-glutamate

or D-aspartate as the sixth ensures high binding. From this information we have constructed a model for the best binding peptides in which the pattern of amino acids follows this sequence: X-A/a-X-A/a-X-a-X-a. (X) can be any amino acid and is often hydrophobic, (A) is L-glutamate or L-aspartate and (a) is D-glutamate or D-aspartate.

Example 6 Preferred examples of TSG-6 binding peptides

Tumor necrosis factor (TNF) stimulated gene-6 (TSG-6) is an HA binding protein whose carbohydrate binding domain is a member of the link module consensus family of which CD44 is also a member(Bajorath et al., 1998). TSG-6 is present in higher than normal concentrations at sites of joint and cartilage inflammation (Maier et al., 1996) and is thought to mediate some of the pathological conditions of arthritis. The structure of the HA binding domain was solved using NMR and is the only structure presently available of an HA binding domain (PDB ascension # 1TSG) (Kohda et al., 1996).

Using TSG-6 as a target protein we screened our random library against this protein using the same experimental methods described previously. We performed this experiment at two different pH solutions: pH 5.7 and 7.5. This is because we wanted to compare neutral pH conditions to that of conditions where maximal HA binding occurs (Parkar et al., 1998). The following sequences were found:

pH 5.7
GYYFNVAM
WAYNFLVM
TQSLNNHM
WWPFINAY

WWKADMVG
WWPFINAY
MALQLPYY
IIYEEFFV
ISINNRWY
VTPPVYFT

pH 7.5
QIRNGWFW
SWWFGPLA
GDWEQILT
PAGFGWNL
NMRFNIEN
QMTFFDGV

While these sequences are different from those found in the screening experiment using GST-P1 as the target protein, there are similarities. There is a motif of double hydrophobic amino acids in these peptides.

Although the present invention has been fully described herein, it is to be noted that various changes and modifications are apparent to those skilled in the art. Such changes and modifications are to be understood as included within the scope of the present invention as defined by the appended claims.

WHAT IS CLAIMED IS:

1. An HA mimic comprising a polypeptide comprising alternating acidic / non-acidic residues, wherein said mimic binds to the hyaluronic acid binding domain of the receptor for hyaluronan mediated mobility.

2. An HA mimic of claim 1, wherein said polypeptide is four to 15 residues in length.

3. An HA mimic of claim 2, wherein said residues include at least one dextrorotatory residue.

4. An HA mimic of claim 1, comprising an amino acid sequence selected from the group consisting of: SEQ ID NO 1; SEQ ID NO 2; SEQ ID NO 3; SEQ ID NO 4; SEQ ID NO 5; SEQ ID NO 6; SEQ ID NO 7; SEQ ID NO 8; SEQ ID NO 9; SEQ ID NO 10; SEQ ID NO 11; SEQ ID NO 12; SEQ ID NO 13; SEQ ID NO 14; SEQ ID NO 15; SEQ ID NO 16; SEQ ID NO 17; SEQ ID NO 18; SEQ ID NO 19; and SEQ ID NO 20.

5. An HA mimic of claim 1, comprising a homologue of an amino acid sequence selected from the group consisting of: SEQ ID NO 1; SEQ ID NO 2; SEQ ID NO 3; SEQ ID NO 4; SEQ ID NO 5; SEQ ID NO 6; SEQ ID NO 7; SEQ ID NO 8; SEQ ID NO 9; SEQ ID NO 10; SEQ ID NO 11; SEQ ID NO 12; SEQ ID NO 13; SEQ ID NO 14; SEQ ID NO 15; SEQ ID NO 16; SEQ ID NO 17; SEQ ID NO 18; SEQ ID NO 19; and SEQ ID NO 20, wherein said homologue binds to the hyaluronic acid binding domain of the receptor for hyaluronan mediated mobility.

6. A nucleic acid compound comprising a nucleic acid which encodes an amino acid sequence selected from the group consisting of: SEQ ID NO 1; SEQ ID NO 2; SEQ ID NO 3; SEQ ID NO 4; SEQ ID NO 5; SEQ ID NO 6; SEQ ID NO 7; SEQ ID NO 8; SEQ ID NO 9; SEQ ID NO 10; SEQ ID NO 11; SEQ ID NO 12; SEQ ID NO 13; SEQ ID NO 14; SEQ ID NO 15; SEQ ID NO 16; SEQ ID NO 17; SEQ ID NO 18; SEQ ID NO 19; and SEQ ID NO 20; and a homologue of an amino acid sequence selected from the group consisting of: SEQ ID NO 1; SEQ ID NO 2; SEQ ID NO 3; SEQ ID NO 4; SEQ ID NO 5; SEQ ID NO 6; SEQ ID NO 7; SEQ ID NO 8; SEQ ID NO 9; SEQ ID NO 10; SEQ ID NO 11; SEQ ID NO 12; SEQ ID NO 13; SEQ ID NO 14; SEQ ID NO 15; SEQ ID NO 16; SEQ ID NO 17; SEQ ID NO 18; SEQ ID NO 19; and SEQ ID NO 20, wherein said homologue binds to the hyaluronic acid binding domain of the receptor for hyaluronan mediated mobility.

6a. An HA mimic comprising a polypeptide comprising double hydrophobic residues, wherein said mimic binds to the hyaluronic acid binding domain of the TSG-6.

7. An HA mimic of claim 6a, which is selected from the group consisting of: GYYFNVAM; WAYNFLVM; TQSLNNHM; WWPFINAY; WWKADMVG; WWPFINAY; MALQLPYY; IIYEEFFV; ISINNRWY; VTPPVYFT; QIRNGWFW; SWWFGPLA; GDWEQILT; PAGFGWNL; NMRFNIEN; and QMTFFDGV, or a homologue thereof, wherein said homologue binds to hyaluronic acid binding domain of the TSG-6.

8. A method to affect a hyaluronidase-mediated biological response in a patient in need of such affecting , comprising administering an HA mimic of claim 1.

9. A method to inhibit hyaluronidase activity in a patient in need of such inhibition, comprising administering an HA mimic of claim 1.

10. A method to bind receptors that bind hyaluronic acid, comprising introducing an HA mimic of claim 1.

11. A method to mimic hyaluronic acid in a biological system, comprising introducing an HA mimic of claim 1.

12. A method to affect cell signalling associated with hyaluronic acid/hyaluronic acid receptor interactions, comprising introducing an HA mimic of claim 1.

13. A method to treat hyaluronic acid-associated disease in a patient in need of such treatment, comprising administering an HA mimic of claim 1.

14. A method of claim 13, wherein said disease is selected from the group consisting of: inflammation; tumor angiogenesis; skin disease; bone disease; wound healing;osteoarthritis; rheumatoid arthritis; infectious disease; immune disease; and cardiovascular disease.

15. A method to inhibit metastasis in a patient in need of such inhibition, comprising administering an HA mimic of claim 1.

16. A method to inhibit fertilization in a patient in need of such inhibition, comprising administering an HA mimic of claim 1.

17. A method to introduce an agent into a cell which binds hyaluronic acid, comprising administering the agent in conjunction with an HA mimic of claim 1.

18. A method to isolate peptides that mimic a ligand's binding to a receptor comprising the steps of:

(a) preparing a random library of peptides and binding said library to a bead;

(b) placing the library in contact with the receptor under conditions for binding;

(c) washing off unbound peptides;

(d) contacting the washed bead-bound library with anti-receptor antibody;

(e) detecting and selecting beads having antibody bound thereto;

(f) eluting the antibody;

(g) repeating steps (a) through (f) and then repeating steps (a) through (c), and then incubating the beads in the presence of receptor prior to adding anti-receptor antibody;

(h) detecting and selecting beads that did not bind antibody;

(i) determining the sequence of the bead-bound peptide.

19. An HA mimic comprising the sequence:

$$X\text{-}A/a\text{-}X\text{-}A/a\text{-}X\text{-}a\text{-}X\text{-}a_1$$

wherein (X) can be any amino acid, (A) is L-glutamate or L-aspartate and (a) and (a_1) are D-glutamate or D-aspartate.

20. An HA mimic of claim 19, wherein X is hydrophobic.

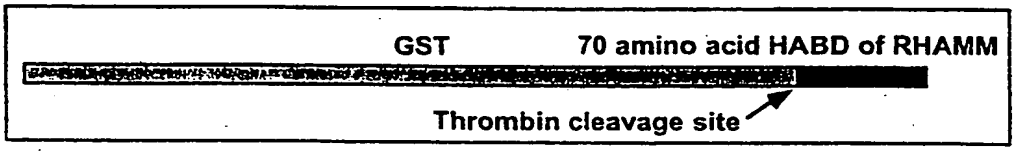
21. An HA mimic of claim 20, wherein a_1 is D-glutamate.

Figure 1

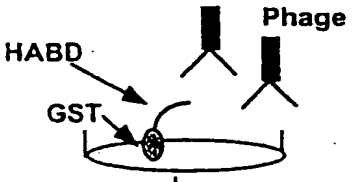
Phage Screened for Peptides that Bind to the HA Binding Domains of RHAMM-1v4

fUSE-5 phage hosting random 15 amino acid peptides were screened against the HA binding domain of RHAMM 1v4.

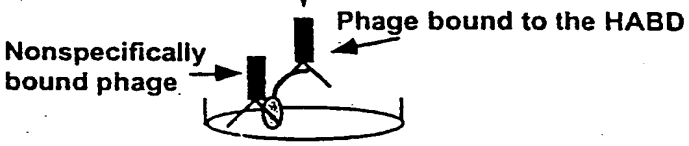
METHOD



Apply phage to immobilized GST-HABD

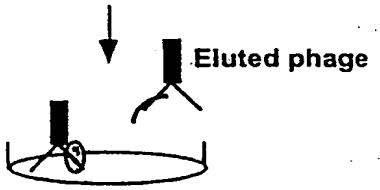


Wash away unbound phage, but nonspecific binders remain.

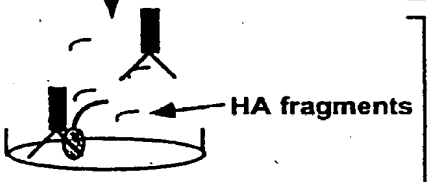


Treat with thrombin to elute only phage bound to HABD.

Repeat two times with phage from previous round.



Final Screen:
No thrombin elution, rather elute phage with HA fragments.



SEQUENCES FOUND

HA2	GVCNADFCWLPAVVV	HA6	HWCLPLLACDTFARA
HA3	SASPSASKLSLMSTV	HA7	YRVYLSVVHNSVLPS
HA4	IPPILPAYTLLGHPR	HA8	PHXRPVVSASSILPV

Scott, J.K. & Smith, G.P., *Sci nc* , 249:386-390 (1990)

Figur 2

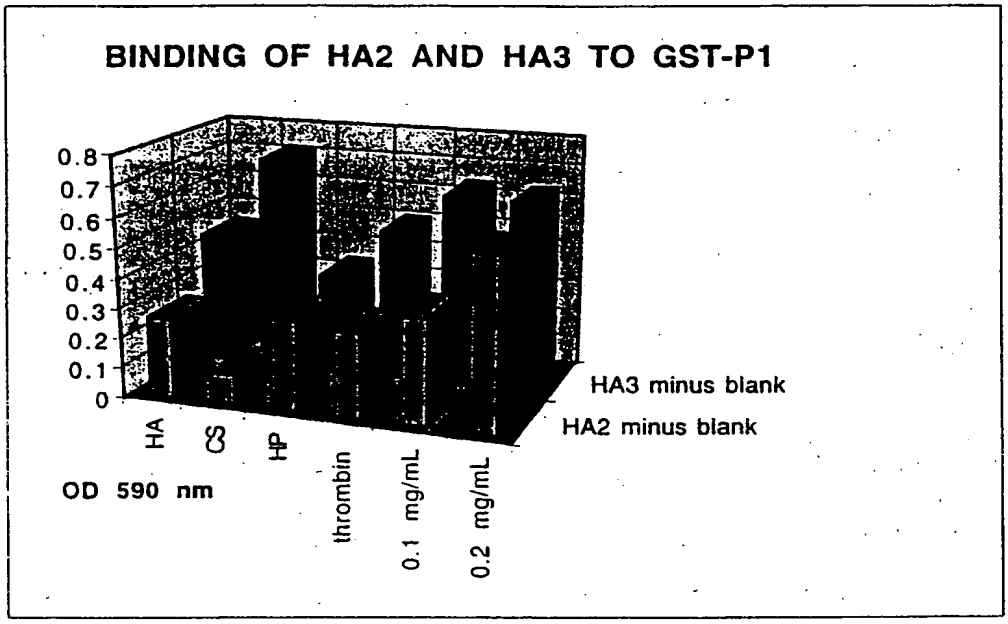


Figure 1. The lanes marked HA, CS and HP have been preincubated with 1.5mg/mL hyaluronic acid (HA), chondroitin sulfate (CS) and heparin (HP). These wells have 0.15mg/mL HA2 or HA3 in them.

Figur 3

Affinities of Peptides that Bind to the HA Binding Domain

The peptides HA2 and HA3 were synthesized with an N-terminal biotin, and evaluated for their binding properties to the HA binding domains from which they were screened. In a solid phase binding assay, in which GST-P1 is immobilized in 96 well plates the following data was obtained.

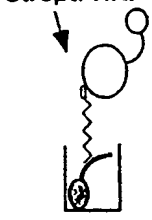
Furthermore, it is noted (data not shown) that HA fragments (20-60 kD) and chondroitin sulfate (60 kD) can compete with the peptides for binding.

GENERAL METHOD

GST-HABD immobilized in a microplate well

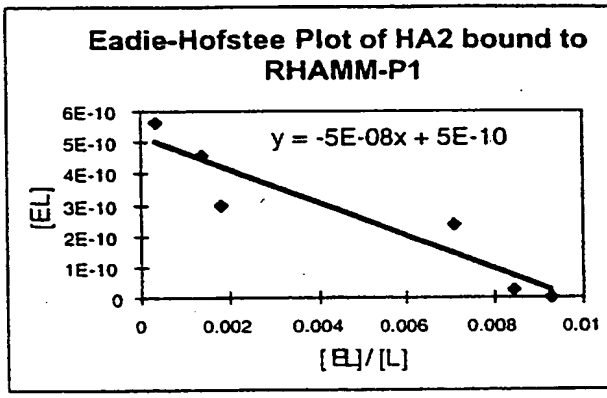
Biotinylated peptide is applied.

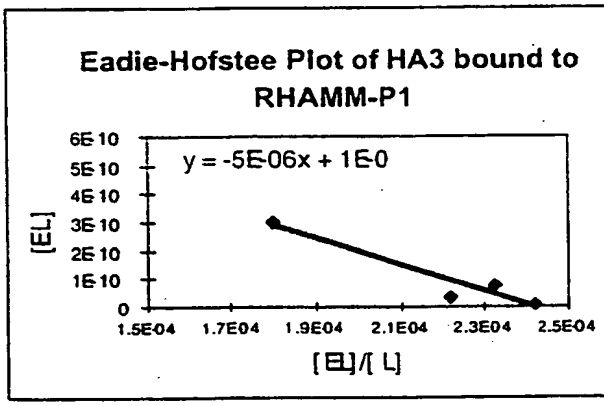
Detect bound ligand using streptavidin-HRP



Read plate at 590 nm

HA2 $K_d = 5 \times 10^{-8}$ HA3 $K_d = 5 \times 10^{-6}$





Figur 4

Amino Acid	Abbreviations Three Letter	One Letter
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Asparagine or aspartic acid	Asx	B
Cysteine	Cys	C
Glycine	Gly	G
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glutamine or glutamic acid	Glx	Z
Histidine	His	H
Isoleucine	Iso	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

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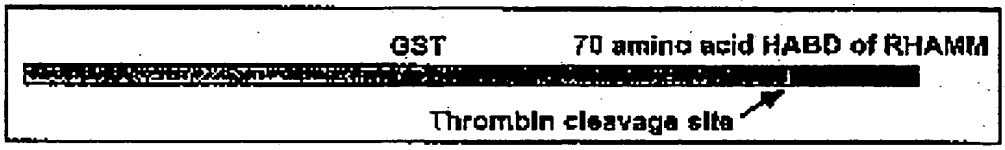
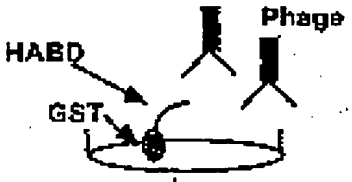


Apply phage to immobilized GST-HABD



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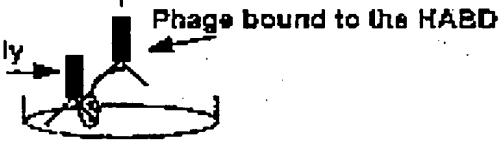

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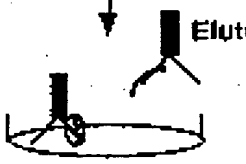

Final Screen:
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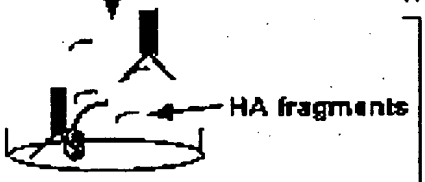

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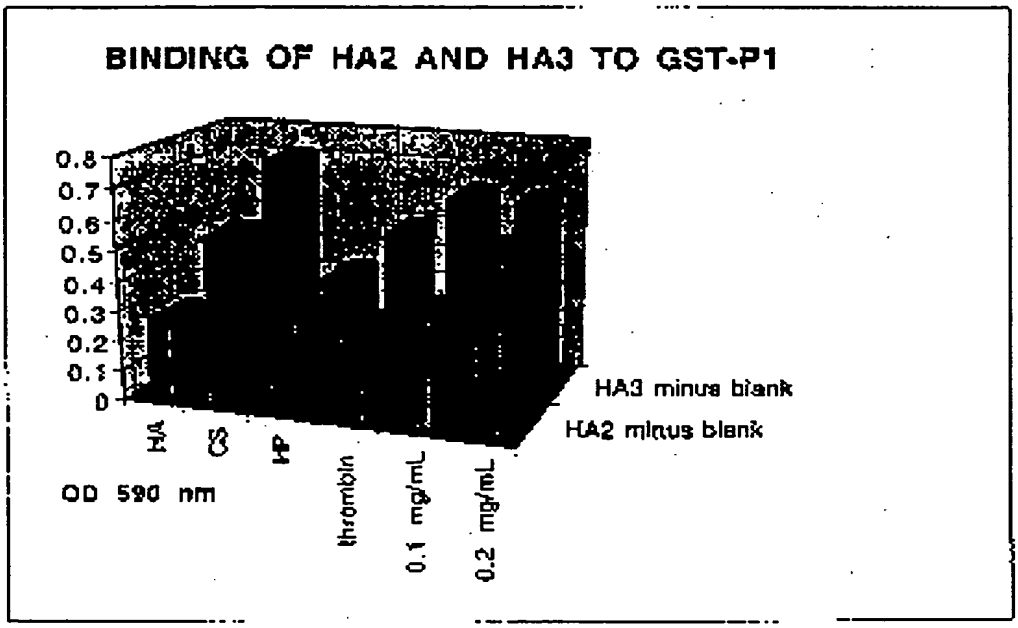

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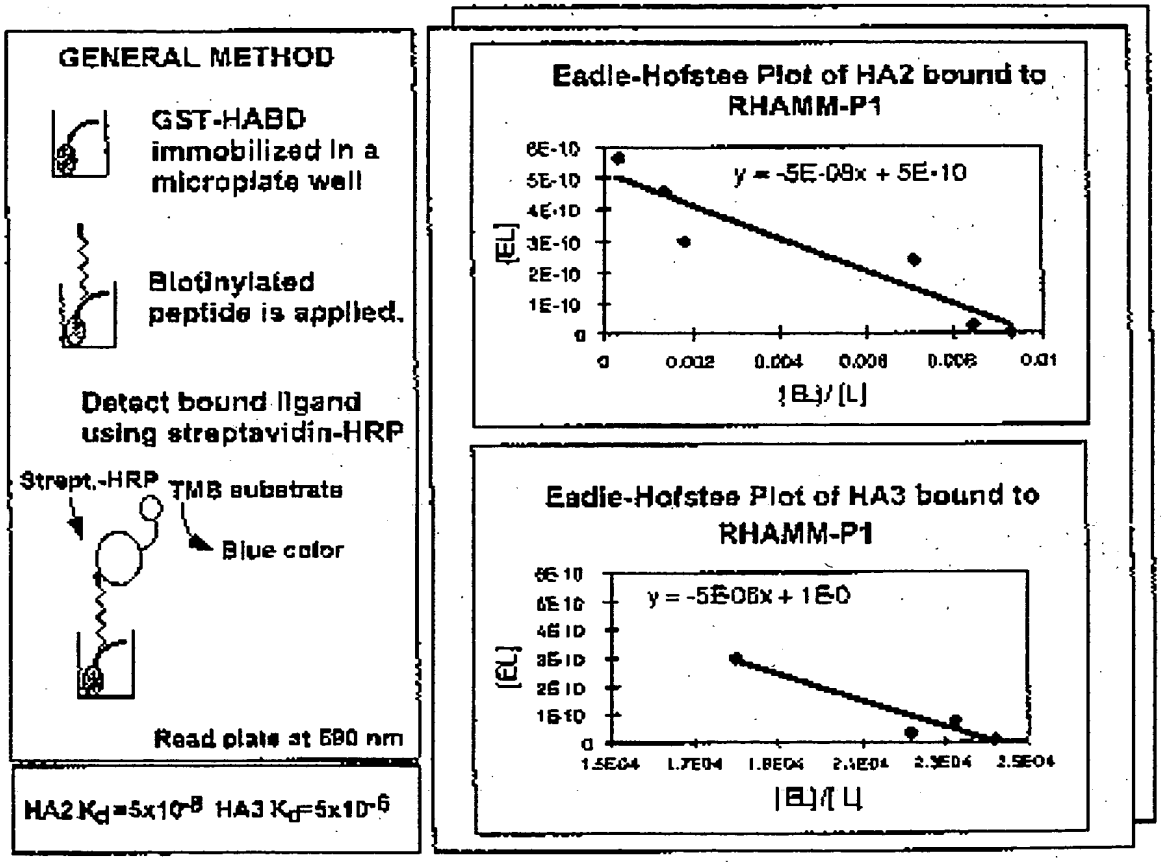

Figure 4

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Glutamic acid	Glu	E
Glutamine or glutamic acid	Glx	Z
Histidine	His	H
Isoleucine	Iso	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V